Temporal dynamics of malarial parasites infecting common yellowthroats (*Geothlypis trichas*)

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Studies of avian malarial parasites generally assume temporal community stability to justify the pooling of several years' worth of field data. Examining the composition of a community of *Plasmodium* parasites infecting common yellowthroats (*Geothlypis trichas*) from 2008-2012 and 2015-2020, we observed drastic longitudinal changes in lineage prevalence challenging the accuracy of pooled-year parasite studies. The prevalence of *Plasmodium* infections increased throughout our 11-year study, primarily driven by the rise of the p06 lineage from 2015-2020. We found p06 infections to be chronic, high-load, and benign relative to p04, the other major lineage in our observed parasite community, allowing p06 to spread rapidly throughout the common yellowthroat population. Upon subjecting experimental p04/p06 mixed infection samples to the molecular lineage identification techniques used in our study, we detected the presence of preferential amplification for p06, a methodological artifact accounted for in our analysis but with major implications for similar studies.
Keywords: *Plasmodium*, parasite community, temporal dynamics, preferential amplification.

Avian parasites and diseases, once considered unimportant for explaining population fluctuations, have been studied in increasing detail over the past half century to understand host population dynamics and evolution (Bensch et al. 2007). Blood parasites, due to their impact on wild birds, are commonly analyzed using molecular approaches to identify the prevalence of specific parasite lineages, affording a comprehensive examination of parasite community composition across space and time (Bensch et al. 2007; Walther et al. 2014). *Plasmodium* and closely related malarial parasites, *Haemoproteus* and *Leucocytozoon*, are most frequently examined due to their similar life cycles which require vertebrate hosts for asexual reproduction and dipteran vectors for sexual reproduction and sporogony (Walther et al. 2014). Most studies focus on the spatial distribution of the aforementioned haemosporidian genera and geographic factors contributing to their success and abundance. Starkloff et al. (2020), for example, identified latitudinal and altitudinal effects on haemosporidian prevalence and diversity in North America, and Harrigan et al. (2014) evaluated how haemosporidian diversity is related to elevation, temperature, and ecological heterogeneity in the Andes. Both studies expressed the sentiment that macroecological patterns of hosts contributing to biological richness have been well researched while patterns in parasite ecology remain comparatively unfamiliar, emphasizing the importance of spatial parasite analyses (Harrigan et al. 2014; Starkloff et al. 2020). Furthermore, the geographic structuring and distribution of avian parasites can be studied to understand host-parasite coevolution and the connectivity of parasites and hosts with regards to migration and dispersal (e.g., Fallon et al. 2006; Pagenkopp et al. 2008; Jenkins et al. 2011).

Due to the cumbersome nature of mist netting, blood sample collection, and molecular analysis of infecting parasites, data sets are often limited, resulting in spatial studies of parasite community composition that pool several years' worth of samples (e.g., Fallon et al. 2006; Pagenkopp et al. 2008; Harrigan et al. 2014; Starkloff et al. 2020). This decision is made under the assumption that parasite communities are relatively fixed over time, a phenomenon demonstrated by *Plasmodium* and *Leucocytozoon* infecting Berthelot's pipits (*Anthus berthelotti*; Spurgin et al. 2012), *Plasmodium* in blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*; Lachish et al. 2013), and *Plasmodium* and *Parahaemoproteus* in tufted titmice (*Baeolophus bicolor*; Fast et al. 2016). However, parasite lineages can undergo temporal turnover and replacement as documented by a community of malarial lineages infecting Swedish willow warblers (*Phylloscopus trochilus*; Bensch and Åkesson 2003) and several passerine hosts in insular communities (Fallon et al. 2004). Temporal variation can also appear as cyclical prevalence fluctuations as demonstrated by *Haemoproteus* and *Plasmodium* lineages infecting great reed warblers (*Acrocephalus arundinaceus*) in Sweden (Bensch et al. 2007). Such patterns call into question conclusions developed from pooled-year studies and highlight the significance of spatially fixed, multi-year studies of parasite communities to further understand the longitudinal behavior of parasites among a variety of host species and ecological conditions.

In the present study, we investigate the prevalence of lineages within a community of *Plasmodium* parasites infecting common yellowthroats (*Geothlypis trichas*) breeding in Saratoga County, NY, USA from 2008-2012 and 2015-2020. We identify temporal patterns and possible ecological explanations for changes in lineage prevalence over time, considering both sides of the host-parasite arms race in which the host may evolve to better defend against a certain lineage or a certain lineage may become more virulent or harmful to the host relative to other lineages. We offer a host-first perspective of infection persistence by assessing the ability of males to clear infections of a given lineage. We also gauge infection persistence from the parasite's point of view by examining the parasitemia of infections from given lineages. Furthermore, the lineages' differential effects on host survivorship and host health are analyzed, the latter of which was measured using weight, hematocrit, and white blood cell density. To ensure the accuracy of our lineage identification methodology, we tested for preferential lineage amplification, a phenomenon observed by Bernotiene et al. (2016) and Valkiūnas et al. (2006).

Methods

Fieldwork

Every summer, from 2008-2012 and 2015-2020, male common yellowthroats defending territories in Saratoga County, NY (43°10'24.6''N, 73°53'19.7''W) were captured in mist nets. We sampled from a total of three sites, STAB, which was used throughout all 11 years; PCOR, used from 2008-2012; and PONDS, from 2015-2020. Upon capture, males were sampled to obtain a blood smear and a <30μL blood sample, which was stored in a heparinized capillary tube and later centrifuged for 10 minutes to determine hematocrit. Packed erythrocytes were lysed in Queen's lysis buffer (Seutin el al. 1991) and stored at 4°C until whole genomic DNA was extracted with a Qiagen DNeasy blood and tissue kit. The captured males were released after being measured for multiple morphological characteristics, including weight (to nearest 0.1g).

Infection status and lineage identity of malarial parasites

Males were screened for *Plasmodium* infection by using the nested PCR methodology developed by Hellgren et al. (2004) to amplify the parasite's cytochrome *b* gene. HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') (I is a universal base, inosine) and HaemNR3 (5'-ATAGAAAGATAAGAAATACCAT-TC-3') were used as the first primer set to amplify general avian malaria blood parasites, yielding a product subsequently amplified with HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') to screen for the presence of *Plasmodium* or *Haemoproteus*. PCR products were electrophoresed on a 1% argarose gel with 80 volts for 30 minutes. Negative samples were run two additional times to confirm the absence of an infection. PCR products from infected birds were isolated using ZymoClean kits before being sent to the DNA Analysis Facility on Science Hill at Yale University to be sequenced. Electropherograms were read using 4Peaks and compared to GenBank in order to identify the lineage (species) of *Plasmodium* infecting each male. Electropherograms with strong secondary peaks indicated coinfection with multiple *Plasmodium* lineages. Secondary infections were identified to lineage by manually editing electropherograms and comparing the resulting sequence to GenBank.

Blood measurements

Blood smears were examined at 1000x using an Olympus CX43 microscope to quantify white blood cells (WBCs) per 1000 red blood cells (RBCs) and parasitemia (parasites per 1000 RBCs). A total of 4000 erythrocytes were examined per male.

PCR bias

The observed parasite community was primarily composed of the *Plasmodium* lineages, p04 and p06 (see Results below). Samples with single p04 or p06 infections and a known parasitemia were used to generate experimental mixtures of p04 and p06 DNA. Specifically, we examined the ability of our PCR technique to reveal the presence of both parasites when ratios of p06:p04 ranged from 1:2 to 32:1. Experimental mixtures were evaluated with DNA amplifying and sequencing methods described above.

Statistical analyses

Pooling data from 2008-2012 and 2015-2020, binomial logistic regressions were run in SPSS with year, Julian date (Jdate), and site of capture as fixed variables and male ID as a random effect to model the likelihood of infection with any *Plasmodium* lineage and separately, infection with p06. A combination of simple linear and polynomial regressions were used to assess cyclical patterns of prevalence and correlated changes in abundance among lineages. Taken together, these models allowed us to describe the factors contributing to changes in disease prevalence over the 11-year study period.

To examine the rise of p06 during 2015-2020, we assessed the effect of *Plasmodium* lineage on parasitemia, host health indicators (weight, hematocrit, and WBC density), and presumed survivorship (host is located the following year), using mixed effect multivariate models. Parasitemia and WBC density were zero-rich and were transformed using log(x+1) and hematocrit was arcsine square root-transformed. Information regarding the lineage of a given male's infection (p04, p06), if any, was included as a categorical variable. We excluded males with multiple infections (CO) and males infected by lineages other than p04 or p06 (OTH) because of their limited sample size; CO males accounted for 24 of the 269 total males (8.92%) and OTH males, infected by one of seven rare lineages, accounted for 11 males (4.09%). Uninfected (UNI) males were removed when running regressions for parasitemia since uninfected males do not have any parasites. Controlling for year, Jdate, and site, regressions were initially run with male ID as a random effect and InfStatus, InfStatus*year, InfStatus*Jdate, and InfStatus*site as fixed effects. In the presence of an insignificant interaction term, the interaction term with the largest p-value was removed and the regression was run until all remaining interaction terms were significant or no interaction terms remained.

Further regressions were run using the same methodology to test the significance of Jdate*year, Jdate*site, and year*site to screen for underlying artifacts of data collection that could potentially alter any lineage-specific *Plasmodium* effects identified by the models described above (see supplementary materials Table S1).

We also ran similar multivariate models including parasitemia and parasitemia*InfStatus as fixed effects to control for the effects of parasite load on a lineage-specific infection, the latter of which was removed if insignificant.

All aforementioned regressions were repeated using a dataset including all males during 2015-2020 to test the robustness of our results and the consequences of adding CO and OTH males (see supplementary materials Table S1-S3).

Results

Over the 11 years considered in this study, we detected 404 *Plasmodium* infections in 369 of the 433 total males. Of the 404 infections, 390 were identified to lineage (96.53%). The *Plasmodium* community was primarily comprised of p04 and p06, accounting for 161 (41.28%) and 181 (46.41%) of the 390 infections identified to lineage, respectively. Overall infection prevalence ranged from 69% in 2009 to 97% in 2018; p06 prevalence ranged from zero in 2011 to 71% in 2019 and p04 from 20% in 2019 to 72% in 2012 (Fig. 1).

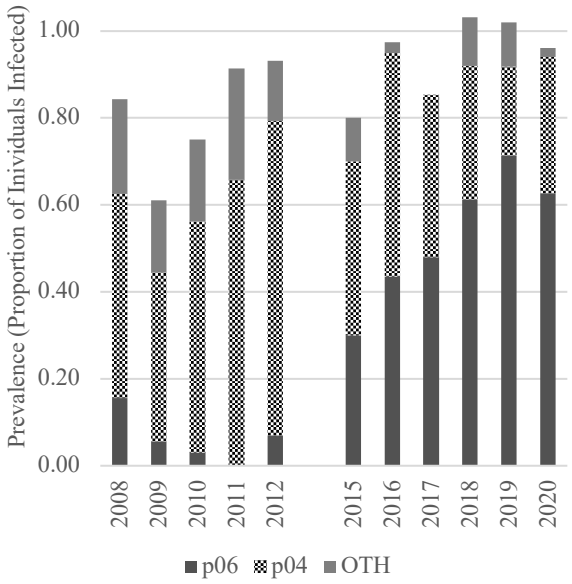

Figure 1. Prevalence of *Plasmodium* lineages infecting common yellowthroats in Saratoga Springs, NY.

Simple linear regressions model the rise in overall prevalence (Fig. 2a; β=0.019, p=0.0025, R^2=0.66) and p06 prevalence Fig. 2b [solid line]; β=0.059, p<.0001, R^2=0.86). Binomial logistic regressions indicated an 18.3% increase in the probability of a *Plasmodium* infection and a 54.7% increase in the probability of p06 infection per year for a given male (Table 1). The change in p06 prevalence over time can also be modeled by a cubic regression (Fig. 2b [dotted line]; β_1=19197, β_2=9.53, β_3=-0.0016, p<0.0001, R^2=0.99).

Table 1. Binomial logistic regressions modelling for infection probability using samples from 2008-2020.

	Year			Jdate			Site		n
	F	p	e^{β}	F	p	e^{β}	F	p	
Plas	9.692	0.002	1.183	3.068	0.081	0.985	0.307	0.736	433
p06	53.043	0.000	1.547	0.149	0.700	0.996	3.625	0.027	419

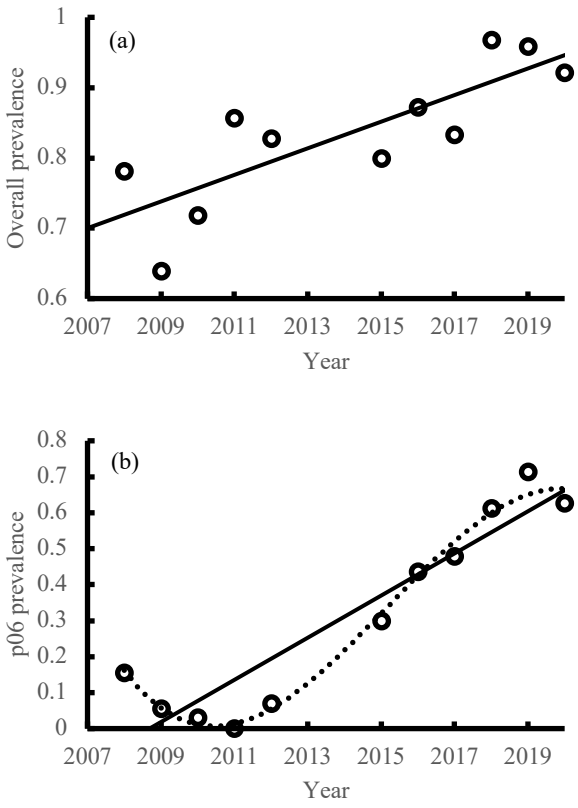

Figure 2. Prevalence of (a) overall and (b) p06 infections among males sampled from 2008-2012 and 2015-2020.

When examined temporally, the prevalence of p04 and p06 negatively covary such that a rise in p06 correlates with a decline of p04 (Fig. 3a). The prevalence of p06 can be modelled to depend on the prevalence of p04 by a simple linear regression (Fig. 3b; β=-0.46, p=.0033, R^2=0.63). P04 was drastically higher in prevalence than p06 in 2008-2012 but becomes the less prevalent strain as of 2017. This apparent replacement of p04 by p06 is most likely an artifact caused by the preferential amplification of p06 in our parasite identification methodology (see below). This detection bias almost certainly resulted in an underestimation of p04/p06 coinfection (CO) frequency, where a male is infected with p04 and p06 simultaneously, and an overestimation of single p06 infection frequency, artificially reducing p04 prevalence and calling into question the negative covariance of p04 and p06 (Fig. 3b). Due to the extreme sensitivity of our lineage identification methods with regards to identifying p06 infections, the rise in p06 prevalence over time remains accurate and is even strengthened by an assurance that p06 can be detected in coinfections with p04 in excess.

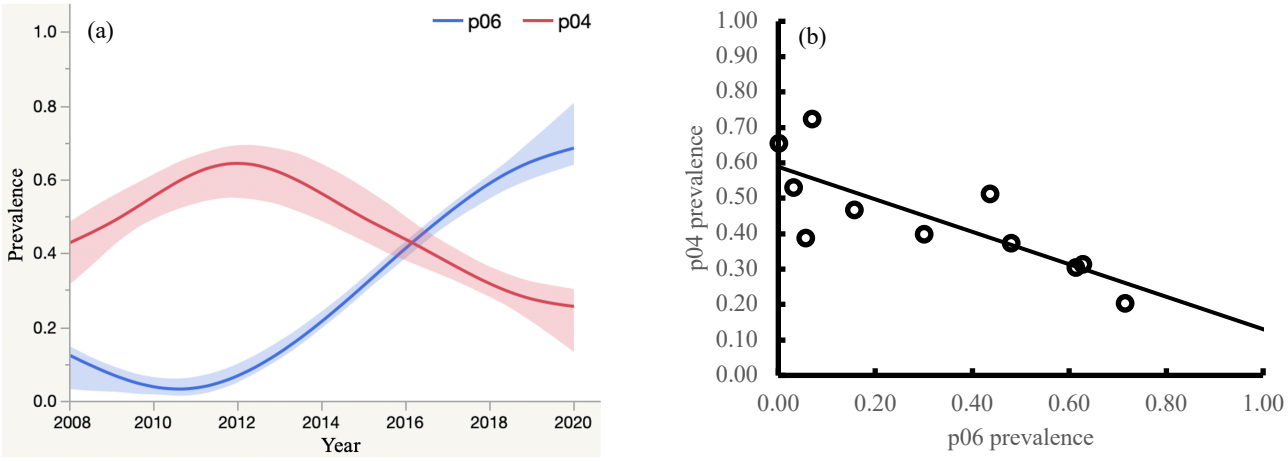

Figure 3. (a) Temporal trends of p04 and p06 prevalence and (b) the relationship between p04 and p06 prevalence pooling data over all years.

PCR bias

During 2015-2020, the proportion of males infected with p06 that also had a p04 infection (coinfection) was significantly lower than the proportion of males without p06 that had a p04 infection for all years except 2016 (χ^2>6.378, p<0.0116). Coinfections may be underrepresented due to a competitive exclusion of lineages within hosts or a PCR bias favoring the detection of p06 in mixed infections. Pooling over 2016-2019, the mean parasitemia of coinfections was 0.0064, compared to 0.0023 for p04 and 0.022 for p06 (Fig 4), suggesting that preferential amplification was likely since p04/p06 coinfections are detected only when the infecting strains have a lower parasitemia than the mean p06 infection. As a result of the hypothesized PCR bias, a coinfection that contains p06 at its average parasitemia would be identified as a single p06 infection.

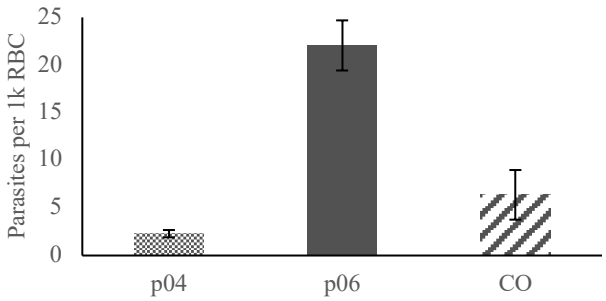

Figure 4. Mean parasitemia for p04, p06, and CO pooled during 2016-2019.

We determined the extent of bias towards p06 in our lineage detection methods by altering the concentration of p06 with regards to p04 in experimental samples that were subjected to PCR. P04 was only detected in experimental mixtures with p04 in excess; when p06:p04 was 1:2, p04 was detected in 2 of 9 trials (22%), and at 1:1.5, we detected p04 in 3 of 10 trials (30%). Five trials were run for mixtures with p06 in excess (2.1:1, 4.2:1, 12.3:1, 31.5:1); all 20 trials resulted in no detection of p04. By taking the parasitemia of a p04 infection and creating parasitemia ratios with all other p06 infections, and repeating this process for every p04 infection, we examined the distribution of p06:p04 ratios that would have resulted if the observed single infections cooccurred in the same bird. Pooling all four years, our PCR method would have been able to detect only 80 of the 868 (9.22%) theoretical mixed infections.

Factors contributing to p06 invasion

A longitudinal analysis of p04 and p06 infections showed that 18 of the 24 (75%) initial p04 infections persisted to the following year during 2008-2012, similar to the persistence rate of p06 during 2015-2020 in which 23 out of 26 infections persisted (88.46%). We could not evaluate the persistence of p04 infections after 2015 due to increasing prevalence of p06 and likelihood of detection bias (p06 favored in mixed infections.

A linear regression found infection status to be a significant fixed effect for parasitemia (Table 2; Fig. 5) with pairwise comparisons indicating p06 to have a greater parasitemia than p04 (p=0.000). Parasitemia increased with the year of sample collection (Table 2; Fig. 5).

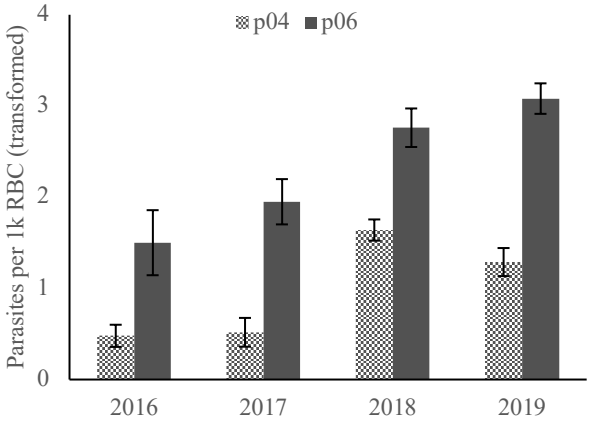

Figure 5. Parasitemia of p04 and p06 separated by year of sample.

Table 2. Mixed effect multivariate models for parasitemia and host weight, hematocrit, WBC, and survivorship.

		InfStatus		Year		Jdate		Site		n
		F	p	F	p	F	p	F	p	
Linear models	**Weight**	3.590	0.029	7.692	0.006	18.412	0.000	7.112	0.008	222
	Hematocrit[A]	0.125	0.882	0.632	0.431	7.411	0.007	15.696	0.000	212
	WBC	2.379	0.096	0.055	0.815	6.223	0.014	0.062	0.803	150
	Parasitemia	54.970	0.000	16.149	0.000	1.576	0.212	0.864	0.354	136
Binomial logistic model	**Survivorship**[B]	2.632	0.075	6.163	0.014	5.651	0.019	0.302	0.583	176

[A] Hematocrit had an InfStatus*site term (F=3.240, p=0.041).
[B] e^{β} for year and Jdate was 0.538 and 0.947, respectively.

Linear regression solving for host weight showed infection status to be a significant fixed effect (Table 2) with p04-infected males found to be 0.266g heavier than uninfected males (p=0.010). P06 and uninfected males were similar in weight.

Hematocrit varied significantly across sites (Table 2), and within STAB, hematocrit tended to be greater among uninfected males than p06-infected males with near significance (p=0.074), but in PONDS there were no significant pairwise infection status contrasts.

Infection status approached significance when predicting WBC per 1k RBC (Table 2) with pairwise contrasts revealing p06-infected hosts to have a greater WBC count than p04-infected hosts (p=0.036). Infection status was also nearly significant for host survivorship (Table 2) with p04-infected males less likely to return the following year than uninfected (p=0.035) and, with near significance, p06 males (p=0.081).

When including parasitemia as a controlled effect, the model for weight had no significant effects. Infection status was nearly significant in the model for WBC (Table 3), with p06 infections resulting in greater WBC than p04 infections with near significance (p=0.064). Lineage was also a significant effect for host survivorship (Table 3) with p06-infected males more likely to survive than males with p04 infections (p=0.037).

Models with additional interaction terms and/or the inclusion of OTH and CO samples were qualitatively similar to the patterns described above except for survivorship, which was not predicted by infection status, likely due to the low sample size of OTH and CO males (supplementary materials Table S1-S3).

Table 3. Mixed effect models with parasitemia as a controlled effect.

		Parasitemia		InfStatus		Year		Jdate		Site		n
		F	P	F	p	F	p	F	p	F	p	
Linear models	**Weight**	0.341	0.560	2.101	0.150	0.785	0.377	2.445	0.120	3.052	0.083	134
	Hematocrit	1.734	0.190	0.006	0.939	3.526	0.063	0.130	0.719	9.138	0.003	130
	WBC	0.003	0.956	3.499	0.064	0.035	0.852	7.406	0.007	0.175	0.677	136
Binomial logistic model	**Survivorship**[A]	0.150	0.699	3.918	0.050	5.742	0.018	4.269	0.041	1.051	0.307	134

[A] e^{β} for year and Jdate was 0.504 and 0.936, respectively.

Discussion

We found the blood parasite community infecting local common yellowthroats to be diverse with regards to *Plasmodium* lineage, and to have experienced an increase in prevalence over the 11 years of our study. This increase has been driven more recently, during 2015-2020, by the rise of p06, a lineage which was initially observed in low prevalence during 2008-2012. The p06 lineage, *Plasmodium homopolare*, was genetically and morphologically identified among six families of migratory and resident birds in North and South America by Walther et al. (2014). Walther also found 100% matches for *P. homopolare* sequences with GenBank entries sampled from eastern USA (as far east as Kalamazoo, MI), Central America, and the Caribbean, resulting in uncertainty regarding the geographic origin of this species. The p06 lineage may have long resided in the observed Saratoga parasite community, only to have recently found success due to long-term cycling of lineage prevalence. Another possibility is that p06 is an invasive allopatric lineage rising in prevalence due to host unfamiliarity. It may be that p06 spread east and/or north with the migration of infected overwintering common yellowthroats inhabiting southern USA, the

Carribean, and Central America (Guzy and Ritchison (1990), the latter two locations shown by Walther et al. (2014) to be occupied by birds infected with p06. Furthermore, the observed population of common yellowthroats breeding in Saratoga County, NY, were tracked using geolocators to the south coast USA (CR Freeman-Gallant, personal communication, May 7, 2021), a location shown by Pagenkopp et al. (2008) to be occupied by p06.

P04 once dominated our observed parasite community before decreasing in prevalence with the rise of p06, pointing to patterns of lineage replacement and turnover. However, our analysis of the Hellgren et al. (2004) PCR technique, which revealed minimal detection of p04 in experimental coinfected samples with p04 in excess and no detection of p04 with p06 in excess, made it clear that our interpretation of temporal lineage dynamics must account for preferential primer bias favoring p06. We found that p06 infections have a greater parasitemia than p04 infections, likely indicating that most coinfected males contained an excess of p06 and that such infections could be mis-identified as single p06 infections, artificially reducing the prevalence of p04. Therefore, while PCR bias does not challenge the notion that p06 has risen in prevalence during our 11-year study, it does question the simultaneous disappearance of p04 and patterns lineage replacement and turnover.

Studies which have used the Hellgren et al. (2004) PCR technique or similar DNA identification methods and fail to test for or mention the possibility of preferential lineage amplification potentially ignore methodological artifacts that would alter data. This concern is further supported by Bernotiene et al. (2016), demonstrating the variability in lineage identification accuracy for several primer sets and experimental mixed haemosporidian infections, and Valkiunas et al. (2006), revealing the failures of PCR amplification in detecting mixed infections which can otherwise be identified using microscopy. In the case of Fallon et al. (2006), in which black-throated blue warblers (*Dendroica caerulescens*) were sampled for *Plasmodium* infections across their geographical breeding range, PCR bias is neither tested for nor mentioned as a limitation to the accuracy of their prevalence measurements. The presence of preferential amplification could indicate that their description of the geographic distribution of parasite lineages is not accurate, perhaps confounding the conclusion that lineage does not provide site-specific information. Starkloff et al. (2020), using the Hellgren et al. (2004) technique, addressed the methodological limitations in identifying lineages involved in intra-genus coinfections as a basis for ignoring co-infected samples in their dataset, but failed to discuss the potential for PCR bias to mis-identify coinfections as single infections. Again, this study's conclusions, which depend on lineage prevalence measurements to recognize geographic effects on parasite alpha and beta diversity, could be made insignificant with the presence of preferential lineage amplification. Primer bias for parasite lineage can significantly alter the accuracy of prevalence measurements for less detectable haemosporidian species and must always be considered when using PCR techniques.

Despite the detection of preferential p06 amplification in our study, the observed rise in p06 prevalence during 2015-2020 remains accurate and is even supported by PCR bias, which assures that p06 is almost always detected even in experimental mixed infection samples with p04 in excess. Our examination of p06-infected males in comparison to p04-infected and uninfected males reveal possible factors contributing to the success of p06 in recent years. Firstly, from a parasite-first perspective, p06 infections have a greater parasitemia than p04 infections, indicating for high transmissibility to mosquito vectors (Pigeault et al. 2015) and the potential for an effective means to spread throughout the common yellowthroat population. From a host-first perspective, males infected with p06 did not experience any significant changes in weight and had greater survivorship than p04-infected males, indicating that p06 parasites are less harmful to the host relative to p04 parasites. Finally, despite increasing the WBC count of hosts, p06 infections experienced low clearance rates, similar to that of p04 during 2008-2012. P06 infections appear to be chronic, high-load, and relatively benign, allowing p06 to be highly transmissible and persistent among common yellowthroat hosts and to rapidly rise in prevalence during 2015-2020.

We observed fluctuations in the prevalence of both p06 and overall *Plasmodium* infections over the course of our 11-year study, demonstrating the danger in assuming temporal stability within parasite communities, which several spatial studies rely on when using pooled-year datasets (e.g., Fallon et al. 2006; Pagenkopp et al. 2008; Harrigan et al. 2014; Starkloff et al. 2020). In addition to not mentioning potential PCR bias as described earlier, Fallon et al. (2006) also fails to consider possible temporal variation within studied parasite communities, furthering muddying their proposed conclusions. Harrigan et al. (2014) introduces the concept of temporal variance in their examination of ecological factors contributing to parasite diversity but considers it unlikely under the assumption that potential temporal dynamics of parasite communities under a certain set of ecological conditions would not be significant enough to alter the differences in lineage prevalence along ecological gradients. Harrigan pooled data within a six-year range (1999-2004); in our study, the prevalence of p06 rose from zero to 43.6% within an analogous timeframe (2011-2016). A similar pattern in Harrigan's observed parasite community would drastically alter the observed prevalence of lineages across the sampled ecological gradient. Pagenkopp et al. (2008) deemed temporal variation to be doubtful since two samples taken at the same location 14 years apart revealed insignificant change in the prevalence of a certain lineage, thus ignoring the possibility of cyclical fluctuations in which lineage prevalence rises and falls over time, returning to values previously experienced. Such patterns of cyclical lineage dynamics were observed in Bensch et al. (2007), where the three most common lineages (GRW1, GRW2, GRW4) in the observed parasite community fluctuated with a periodicity of about 3-4 years. The prevalence of GRW2 decreased a couple percentage points when sampling only 1985 and 1999 (14 years apart), giving a false perception that GRW2 remained constant for over a decade. In reality, the prevalence of GRW2 rose and fell dramatically throughout this 14-year period, with a maximum of about 15% and a minimum of 0%, and is nearly identical in 1997, 1999, and 2000

(Bensch et al. 2007). The potential for cyclical variation to be hidden in a comparison of data points taken several years apart undermines Pagenkopp's justification for ignoring possible temporal parasite community dynamics. Starkloff et al. (2020) also used a wide range of sample collection years but attempted to control for such variation by including a random effect that binned the duration of sampling at each site into short (<5 years), mid (5-15 years), and long (>15 years). This assumes that temporal variation in the studied parasite communities is unidirectional and uniform across all sites, again allowing potential cyclical variation to diminish the effectiveness of Starkloff's sampling-duration random effect method.

In addition to having meaningful implications for the effectiveness of pooled-year parasite studies, the temporal dynamics and instability documented in our observed *Plasmodium* community may provide the genetic variance necessary to fuel the good genes model of sexual selection among common yellowthroat hosts, in which females select males based on quality-indicating ornamentation (Andersson 1994). The area of carotenoid-based yellow bibs on male common yellowthroats indicates male quality and is actively selected for by females in the local Saratoga population as seen in both a multi-year field study (Freeman-Gallant et al. 2010) and aviary paired choice trials (Dunn et al. 2008). However, mechanisms maintaining any genetic variation among such hosts have not been discovered. Temporal instability within an infecting parasite community may be critical in this regard if gene-based resistance is parasite-specific (*sensu* Bensch and Åkesson 2003).

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